Differentiation between two distinct classes of viruses now classified as human herpesvirus 6

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ABSTRACT Human herpesvirus 6 (HHV-6) causes exanthem subitum (ES, roseola infantum), a childhood disease characterized by high fever and skin rash. We have analyzed restriction enzyme cleavage patterns of the DNAs of ES viruses isolated from Japan and the United States. The patterns of all the ES viruses were highly conserved, except for variable sequences within the terminal repeat sequences. They resembled closely the restriction enzyme patterns of the Z29 strain of HHV-6 but were distinct from those of the U1102 strain. That all ES isolates were closely related whereas the U1102 patterns were very different suggests that the U1102 strain represents a distinct virus. Moreover, the ES isolates all resembled the Z29 strain and not the U1102 strain with respect to reactivity with HHV-6 monoclonal antibodies. These findings provide evidence for the existence of two distinct classes of viruses previously classified as HHV-6. Whereas the Z29-like viruses are involved in ES infections, the association of the U1102-like viruses with human disease has yet to be determined.

Human herpesvirus 6 (HHV-6) was initially isolated from peripheral blood lymphocytes (PBLs) of AIDS patients and patients with lymphoproliferative disorders (1–6). The virus was found to be ubiquitous in humans, with seroconversion occurring during the first 2 years of life (7–10). Recent reports have shown it to be present also in saliva of healthy adults (7, 11–13). HHV-6 is the causative agent of exanthem subitum (ES, roseola infantum), a childhood disease characterized by spiky fever and skin rash (10, 14). Potential association of HHV-6 with serious disease has been reported in immunosuppressed patients following liver, heart, and kidney transplantations (15–18). Further, it has been suggested that the virus might be associated with cases of infant hepatitis (19, 20).

The U1102 strain, which was isolated from an AIDS patient in Uganda (2), and the Z29 strain, which was isolated from an AIDS patient in Zaire (4), exhibit variations in a number of properties. Although both strains can be propagated in fresh cord blood lymphocytes (CBLs) and PBLs, they differ in their ability to replicate in continuous cell lines (2, 21, 22) and in their reactivity with a number of monoclonal antibodies (mAbs) (23) prionted by Balachandran et al. (23) against HHV-6 strain GS (1). The two strains exhibit pronounced restriction enzyme polymorphism, although all the U1102 and Z29 clones tested cross-hybridized with the heterologous DNAs (24). Finally, restriction enzyme mapping analyses ongoing in several laboratories have revealed pronounced differences (R. Honess and colleagues, National Institute for Medical Research, Mill Hill, London, personal communication; P. Pellet and colleagues, Centers for Disease Control, personal communication; and R. M. Danovich, E.C.S., and N.F., unpublished results).

HHV-7 has been isolated from CD4+ T cells of a healthy individual (24). While it appears to be related to HHV-6, the two viruses exhibit very different restriction enzyme patterns. Moreover, hybridization tests using HHV-6 U1102 and Z29 probes revealed either no homology to HHV-7 DNA or only limited homology, even with large probes, ranging in size from 2 to 12 kilobases (kb) (24).

The present study was undertaken to characterize ES isolates. We found that all the ES isolates resembled the HHV-6 strain Z29 and not the U1102 strain with respect to DNA structure and antigenic reactivity. These results raise questions regarding the classification of HHV-6 isolates.

MATERIALS AND METHODS

Virus isolation and Propagation. The Z29 strain of HHV-6 was obtained from Carlos Lopez (Centers for Disease Control). It was propagated in our laboratory in human PBLs, as described (25). J JHAN cells and the U1102 strain of HHV-6 were obtained from Robert W. Honess (National Institute for Medical Research, Mill Hill, London). The U1102 virus was propagated in J JHAN cells in RPMI 1640 medium containing 10% fetal bovine serum. Strains HST, KDT, KSM, KWG, and SUZ were isolated at the University of Osaka, Japan, from ES patients during the acute phase of the disease. The isolates were propagated (14) first in CBLs and were then transferred to the National Institutes of Health, Bethesda, MD, where they were used to infect phytohemagglutinin-activated PBLs, for the DNA and immunofluorescence analyses.

Strains VW and AW were isolated at the National Institutes of Health, from ES patients in Washington, DC. PBLs obtained from patients during the acute phase of the disease were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 5 µg of phytohemagglutinin per ml, and 32 units of interleukin 2 (Advanced Biotechnology, Silver Spring, MD) per ml. After the appearance of cytopathic effect, each of the viruses was propagated in two separate CBL cultures, yielding virus stocks that were propagated independently from the patients' PBLs.

DNA analysis. DNA was prepared from infected cells labeled with [32P]orthophosphate (25). For the hybridizations, infected-cell DNAs were digested with restriction enzymes, electrophoresed in 0.7% agarose gels, and blotted onto Nytran (Schleicher & Schuell). The probes were labeled in vitro using a 32P-tagged random primer or using digoxigenin-substituted DNA coupled with alkaline phosphatase-conjugated anti-digoxigenin (Genius system, Boehringer Mannheim). The pH9 probe was a gift of Robert W. Honess. It contained the 8.5-kb HindIII fragment of U1102

Abbreviations: HHV, human herpesvirus; ES, exanthem subitum; CBL, cord blood lymphocyte; PBL, peripheral blood lymphocyte; mAb, monoclonal antibody.

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DNA. The pNF102 probe contained a 3.9-kb Sal I fragment of U1102 DNA mapped to the junction between the unique and terminal repeat sequences.

Immunofluorescence Assay. Acetone-fixed infected cells were tested by indirect immunofluorescence assay (21) using mAbs derived and characterized by Balachandran et al. (23) for the GS strain of HHV-6 (1). The mAbs were obtained from N. Balachandran (University of Kansas, Medical Center, Kansas City, KS).

RESULTS

Analyses of Viral DNA from Five Japanese ES Isolates. First we examined the DNAs of five ES isolates from Japan. 32P-labeled DNAs were prepared from cells infected with the ES virus isolates, two variants (A and B) segregated from the HHV-6 strain Z29, the U1102 strain of HHV-6, and the RK strain of HHV-7. Restriction enzyme patterns of the DNAs are exemplified in Figs. 1 and 2 and can be summarized as follows. (i) The cleavage patterns of the DNAs from the ES virus isolates were different from those of HHV-7 DNA. As before (24), HHV-6 and HHV-7 DNAs exhibited pronounced differences in their restriction patterns. For example, in the Sal I and Sst II patterns shown in Figs. 1 and 2, HHV-7 DNA generated few large fragments whereas digestion of the ES DNAs or Z29 DNA or U1102 DNA yielded multiple smaller fragments. (ii) The restriction patterns of the five ES isolates closely resembled those of the Z29 strain whereas very few or no DNA fragments of the five ES isolates comigrated with DNA fragments of the U1102 strain of HHV-6. (iii) While the restriction patterns of the ES isolates and Z29 DNA were generally similar, two types of variations were noted. The first corresponded to variability in a single strain reflecting the loss or gain of a fragment in that strain. For example, in the HindIII patterns, a 4-kb fragment was present in all ES isolates except strain HST. Conversely, a 2-kb fragment was present in the Z29 strain but was absent from the ES virus patterns (Fig. 1, asterisks).

The second type of variations reflected the presence of hypervariable regions in the HHV-6 genome. This hypervariability was clearly apparent in the fragments denoted heterogeneous (Het) in Figs. 1 and 2, as well as in the Kpn I, Dra I, Pst I, BamHI, Bgl II, and Ssp I patterns (data not shown). The sizes of these fragments varied in different strains and they differed also in the A and B variants of the Z29 virus stock. In addition, the Het fragments exhibited variable

Fig. 2. EcoRI and Sst II digests of ES isolates from Japan, the A and B variants of HHV-6 strain Z29, HHV-6 strain U1102, and HHV-7 strain RK. Het denotes the fragments showing hypervariability.

Fig. 1. Sal I, Xba I, and HindIII digests of ES isolates from Japan, the A and B variants of HHV-6 strain Z29, HHV-6 strain U1102, and HHV-7 strain RK. Het denotes the hypervariability in the HindIII patterns. Fragment sizes (kb) shown refer to Z29 HindIII fragments in lane 25. These sizes were obtained from a separate gel in which HHV-6 (Z29) digested DNA was analyzed along with Bgl II and BamHI digests of herpes simplex virus 1 (Justin) DNA, serving as size markers (data not shown). The arrow denotes the 8.5-kb HindIII fragment of U1102 DNA. The 4.0- and 2.0-kb fragments variable in the HST and Z29 patterns are denoted with asterisks.
relative molarities in different DNA preparations of the same strain. These fragments map within the terminal repeats, and the variable molarities reflect different proportions of concatenated and unit-length genomes (unpublished data).

Analyses of ES Isolates from the United States. To test whether the close resemblance of the ES isolates reflected the prevalence of certain HHV-6 strains in Japan, we analyzed two additional ES isolates from Washington, DC. To ascertain that the isolates represented viruses in the original ES samples rather than passenger viruses resident in CBL carrier cultures, each isolate was propagated in two different CBL lineages, generating two independently propagated viral stocks. Virus propagation in fresh CBLs was necessary because the two isolates failed to propagate in continuous cell lines.

For the DNA analyses, CBL were infected with the duplicate stocks (I and II) of the ES isolates from the United States, the KWG ES strain from Japan, the HHV-6 strains Z29 and U1102, and the HHV-7 strain RK. The results are exemplified in Fig. 3 and can be summarized as follows. (i) The cleavage patterns were not affected by virus propagation in CBLs and identical patterns were observed with the duplicate virus stocks (I and II). (ii) The patterns of the American ES isolates were very similar to the Japanese ES isolates. They also resembled the Z29 patterns and not the U1102 patterns. (iii) The two virus strains from the United States exhibited variability in the Het fragments, in similarity with the virus isolates from Japan. The variability appeared to be an inherent property of the strain rather than to have arisen during virus propagation in CBLs.

Hybridizations with Probes Representing Unique and Reiterated Sequences. Unlabeled DNAs were next analyzed by blot hybridizations using two probes chosen to elucidate the variability of HHV-6 strains and to confirm their distinction from HHV-7. The pH9 probe, containing an 8.5-kb HindIII fragment of U1102, represents unique sequences of HHV-6 DNA and, as expected, it hybridized to an 8.5-kb HindIII fragment of U1102 DNA (Fig. 4, lane 10). In contrast, the pH9 probe hybridized to two HindIII fragments (5.4 and 20 kb) of Z29 and the seven ES isolates. The pH9 sequences did not hybridize to HHV-7 DNA (Fig. 4, lane 11) confirming our previous observation that this probe was specific for HHV-6 DNA (24).

The second probe employed in the hybridization analyses was pNF1022 containing a 3.9-kb Sal I fragment of U1102 DNA. This probe maps at the boundary of the left terminal repeat and unique sequences. It hybridizes to fragments arising from the right and left genomic termini, as well as to fragments arising from junctions of HHV-6 DNA concatemers (unpublished results). As seen in Fig. 4, this probe hybridized to the variable EcoRI Het fragments in the 7 ES isolates, Z29 A and B variants, and the U1102 virus. It also hybridized to HHV-7 DNA, confirming that HHV-7 contains sequences homologous to this probe, as previously shown (24). We conclude that the unique sequences contained in the pH9 insert represented relatively nonvariant ES fragments, whereas sequences of the 3.9-kb insert of pNF1022 were overlapping in part with the hypervariable fragments.

Antigenic Relatedness of the HHV-6 ES Isolates. Cells infected with the five ES isolates from Japan, the two ES isolates from the United States, and the HHV-6 strains Z29 and U1102 were fixed with acetone and tested by indirect immunofluorescence assays using mAbs that were derived and characterized by Balachandran et al. (23). All the mAbs reacted with the U1102-infected cells, as previously reported (21), whereas the mAb 4A6, directed against the 180-kDa protein p180, and the 4 mAbs 2D6, 13D6, 2D4, and 3B5, directed against an 82- to 105-kDa glycoprotein, gp82-105 (23), did not react with the cells infected with the ES isolates and the Z29 strain. The ES isolates and the Z29 strain interacted with the remaining four mAbs, including 6A5D5 and 7A2, specific for distinct glycoproteins; 12B3G4, specific for the major capsid antigen; and 9A5D12, which recognizes 41- and 110-kDa proteins (23). Thus, the seven ES strains resembled the Z29 strain and not the U1102 strain with respect to their interactions with the mAbs (Table 1).

**DISCUSSION**

These studies revealed significant differences between the ES/Z29 and the U1102 strains. These results form the basis for the reevaluation of the relatedness of the Z29-like and U1102-like strains and their classification as a single group of viruses. In this context it is useful to review current information regarding the properties of these HHV-6 strains. Although the GS strain, the first HHV-6 isolate (1), was not
included in the analyses, the criteria listed below indicate that GS is closely related to U1102.

Variations in Growth Properties. Although the Z29 and U1102 strains can be efficiently propagated in fresh CBLs or PBLs, they differ in their ability to replicate in continuous cell lines. Thus, U1102 as well as the GS strain can be propagated in continuous cell lines, whereas Z29 is more restrictive (2, 21, 22, 26). It remains to be seen whether this reflects inherent differences in growth properties of the viruses or the selection of variants capable of growth in the cell lines.

Variations in Viral DNA. The full assessment of the genetic relatedness of Z29 and U1102 awaits the complete nucleotide sequencing of their genomes. At present, restriction enzyme patterns offer a valuable tool in studies to distinguish the two strains molecularly and epidemiologically, as has been the case in studies of the classification and epidemiology of other herpesviruses (e.g., refs. 27 and 28). In our studies of HHV-7 (24), we found that although Z29 and U1102 exhibited restriction enzyme polymorphism, they were closely related inasmuch as clones generated from either strain cross-hybridized with the heterologous strain. By comparison, large clones of the U1102 or Z29 DNAs exhibited either no homology or only partial homology to HHV-7 DNA, defining this virus as a separate herpesvirus. Further studies (L.S.W., E.C.S., R. M. Danovich, and N.F., unpublished results) have shown that HHV-6 and HHV-7 are immunologically and molecularly distinct. In the present study we have compared seven ES isolates from Japan and the United States with the Z29 and U1102 strains. Our analysis confirmed and extended previous reports by Pellett et al. (29) and Yamanishi et al. (13) that ES strains from Japan were similar to Z29. Especially striking, however, was the observation that all ES/Z29 isolates were closely related, whereas cleavage of the U1102 DNA yielded many fragments that did not comigrate with the Z29-like virus bands. We conclude that the differences between the Z29 and U1102 strains cannot be explained by pronounced restriction enzyme polymorphism of HHV-6 strains, inasmuch as the ES/Z29 HHV-6 strains were well conserved, yet the U1102 strain was clearly distinct.

Heterogeneity in the fragments representing the terminal sequences of variants present in the Z29 virus stock was first observed by Pellett et al. (as quoted in ref. 30). In our own studies we have segregated such Z29 variants and have characterized structural features responsible for these variations (unpublished results). Several elements contribute to the heterogeneity: (i) stable and inherent variations in the size of the terminal repeats of different virus strains; (ii) different ratios of cleaved and concatameric genomes present in different preparations of viral DNA within a given strain; and

![Table 1. Reactivity of HHV-6 mAbs with cells infected by various HHV-6 strains](https://example.com/table1.png)

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The Z29 and U1102 strains of HHV-6 were tested with ascitic fluids at dilutions of 1:10, 1:100, and 1:1000. The remaining HHV-6 strains were tested with the following dilutions: 1:100 for 2D6, 13D6, 2D4, 3B5, 6A5D5, 12B3G4, and 4A6; 1:1000 for 7A2 and 9A5D12. +, Reactivity; 0, no reactivity; ND, not done.

*Ref. 23.
(iii) additional diffuse low-molarity bands whose nature is unknown.

Agglutine Relatedness and Epidemiology of the Z29-Like and U1102-Like Strains. Assessment of the prevalence of Z29-like and U1102-like viruses awaits the derivation of reagents which enable unambiguous distinction between these strains. Our study has suggested the utility of the pH9D probe of U1102, which was derived by Robert W. Honess and co-workers, or its equivalent clone pZVH14, derived by Josephs et al. (31) from the GS strain, as a potential marker for the classification of HIV-6-related isolates. Thus, the pH9D probe is specific for HIV-6 and had no detectable homology in the HIV-7 genome (24). It hybridized to a single HindIII fragment (8.5 kb) of U1102 DNA but to two HindIII fragments (5.4 and 20 kb) in the ES/Z29 isolates. In fact, variations in restriction enzyme patterns of viral genomes have been previously noted in studies employing the 8.5-kb pZVH14 hybridization (6, 32–34). The entire genome patterns have not been investigated in these studies. It remains to be seen whether the presence of an 8.5-kb HindIII band or 5.4- and 20-kb fragments can be taken as a universal characteristic of the U1102 and Z29/ES like viruses. Furthermore, the mAbs derived by Balachandran et al. (23) can be also used to type the type of HIV-6 as clearly reviewed in Table 1.

Z29-like strains have been isolated from AIDS patients, including the Z29 virus itself, and ES patients (refs. 13 and 29 and this study). The ES isolates obtained by Kikuta et al. (32) were most likely Z29-like, inasmuch as HindIII digestion of DNAs of these viruses yielded two fragments that hybridized with pZVH14 rather than a single fragment. A number of strains recently isolated from healthy individuals, most likely by reactivation from latency in vitro, all resembled the Z29 virus (N.F., G. Katsafanas, E.C.S., and L.S.W., unpublished data). It appears that the viruses infecting young children are Z29-like and that these viruses persist in adults to be potentially reactivated later. It is unclear whether infection with Z29-like virus precludes infection with the U1102-like strains and whether the high prevalence of antibodies in the human population represents both viruses. In fact, the prevalence of U1102-like virus infection in the human population is as yet undetermined and further work is required to derive specific reagents that will allow serological distinction between the Z29- and U1102-like strains. Finally, further consideration should be given to the reclassification of HIV-6 strains; a proposal to this effect has been submitted to the Herpesvirus Study Group of the International Committee for the Taxonomy of Viruses.

This paper is dedicated to the memory of Robert W. Honess (National Institute for Medical Research, Mill Hill, London). We wish to acknowledge his numerous contributions to our studies of the T-lymphotropic herpesviruses. We thank N. Balachandran (University of Kansas Medical Center) for the gift of mAbs. We thank George Katsafanas for his skillful technical assistance. We thank Robert M. Danovitch for helpful discussions.


